FINAL REPORT

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Project Title

"A Histamine Dipstick Test for Spoilage in Fisheries Products"

Grantee: Robert Bateman, University of Southern Mississippi

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Project Summary

The overall goal of this project is the development of a simple and rapid method for determining histamine in processed and fresh seafood products. The approach involves the utilization of an enzyme, an diamine oxidase, which will act on the histamine to release a product, hydrogen peroxide. The peroxide can then be detected with a second enzyme, peroxidase, which converts a colorless dye substrate to a readily observable and quantifiable color. The specific objectives of the proposal are restated below:

I) The basis of the dipstick is the recognition of histamine by a diamine oxidase. The dipstick will be reformulated with recombinant human kidney diamine oxidase substituted for the purified pig kidney diamine oxidase used previously. Two new dyes will also be tested as part of the reformulated dipstick.

II) This second generation dipstick will then be tested with histamine standard solutions and with a series of decomposed tuna steaks. Results will be compared to those obtained using the standard AOAC test for histamine.

III) The laboratory of the PI will coordinate a limited AOAC Peer-Reviewed Method Validation trial with two FDA seafood inspection laboratories.

Project Management

Dr. Robert Bateman served as PI on the project and directed all associated personnel. Graduate student Rachell Booth performed most of the molecular cloning and expression experiments. Undergraduates Eric Williams, Christine Thomas, Candice Harper, Thad Sharp, and David Brown participated in smaller aspects of the project. Williams and Harper participated in the initial cloning experiements, while Sharp and Brown assisted in maintenance of the cell culture.

Summary of Project Results

- We identified an amino terminal protein sequence from the pig kidney diamine oxidase and very similar cDNA sequences from rat and human sources. From a consensus sequence derived from these sources oligonucleotide PCR primers were designed to amplify the entire cDNA sequence. Repeated attempts to amplify the entire coding sequence from cDNA made from mRNA from pig kidney and mouse kidney tissues were unsuccessful.
- Human DAO cDNA was obtained from Dr. Hubert Schwelberger of the Universitaetsklinik fuer Chirurgie in Innsbruck, Austria and incorporated into two different insect (Drosophila) expression vectors, one inducible and one constitutive.
- DAO expression vectors were stably transformed into a Drosophila cell line using the DES system from Invitrogen.
- A copper-inducible secreting expression vector containing the cDNA for a control protein (human pituitary glutaminyl cyclase) was likewise stably transformed into a Drosophila cell line, producing high levels of a control protein in our laboratory.
- Three different assays (liquid enzyme assay, solid phase enzyme assay, quinone recycling assay) were used in an attempt to detect the presence of active diamine oxidase in the media of the cell culture. Only the quinone recycling assay suggested active enzyme, albeit at low levels.
- Attempts to maximize induction of diamine oxidase by varying the concentrations of copper sulfate and induction times again yielded a response with the quinone cofactor assay, but not with the enzyme activity assay.
- We have purified pig kidney DAO by our previous published protocol and reformulated the stick with a new dye and stabilizer. The stick had a pH optimum for histamine of 7.0 and was sensitive to histamine in buffer samples down to 0.03 mM concentration. This was slightly lower than our previous histamine dipsticks, but would still require a larger than available amount of pig kidney diamine oxidase to be commercially viable.

Evaluation

Project goals were not achieved because of the lack of an abundant source of active enzyme. There are several possible reasons that we were unable to successfully express the mammalian diamine oxidase. The most probable one, in my opinion, is that the clone given us by Schwelberger was defective in some way. Another likely cause is simply that my laboratory personnel were not experienced enough at molecular biology techniques to tackle a difficult problem such as this one in which a large, multimeric protein is expressed in a heterologous system. Additionally, problems of stability of diamine oxidases with various cations have recently been published. The report states that freezing of pig kidney diamine oxidase in the presence of Na+ or Li+ surprisingly resulted in complete and irreversible inactivation. (Eur J Biochem 2001 Sep;268(17):4686-97) Although we usually use potassium-containing buffers in working with diamine oxidase, it is quite possible that sodium-containing buffers were used occasionally without the knowledge that they destabilized the enzyme.

Finally, it is both interesting and frustrating to us that a recent paper by Dooley and coworkers at Montana State University describes exactly what we were trying to do. The first two lines of

their abstract are say it all. "Human kidney diamine oxidase has been overexpressed as a secreted enzyme under the control of a metallothionein promoter in Drosophila S2 cell culture. This represents the first heterologous overexpression and purification of a catalytically active, recombinant mammalian copper-containing amine oxidase." (J Biol Inorg Chem 2002 Jun;7(6):565-79) Dooley is a crystallographer who is well known for his work with bacterial amine oxidases. He did not respond to my request for reprints or enzyme samples.

One spinoff of this diamine dipstick work has been to use the dipsticks, which were originally designed for measuring diamines in seafood, to measure diamines in urine. Proliferating cells, such as those found in cancers, spill diamines into the bloodstream and ultimately into the urine. A rapid test for measuring these diamines in urine could act as a rapid diagnostic screen for cancer, or even a test to monitor the efficacy of cancer therapy and provide surveillance for relapse. There are obstacles to overcome with this adaptation of seafood technology to the testing of urine, the primary one being the acetylation of many diamines in the liver, rendering them insensitive to our dipsticks. Addition of a deacetylase has proved promising in our preliminary tests and we are currently optimizing a simple urine cleanup protocol to maximize the dipstick response.

Recommendations for histamine testing in seafood:

The current FDA levels of concern with histamine in tuna are at the borderline of detection with colorimetric dyes. I now believe that a dependable, sensitive field test for histamine in tuna and other seafood will be based on more sensitive detection technology than the enzyme-based colorimetric dipstick in this proposal. Histamine will not be detected with an electronic nose because it is nonvolatile. The most promising technology at the current time for a rapid histamine test appears to be a histamine electrode (Anal. Biochem 2002 304(2):236-43; Anal. Bioanal. Chem 2002 372(2): 276-83; Anal Chem 2002 74(5):44-8; Inflamm. Res 2001 50 Suppl 2:S146-8; Biosci Biotechnol Biochem 2000 64(9):1963-6; Anal Chem 2000 72(10):2211-5) rather than HPLC or CE analysis. We have made and tested carbon paste electrodes containing methylamine dehydrogenase. These successfully detected histamine and other diamines, but the project was abandoned because the electrodes were unstable to storage and had very limited reuse. The recent publications on histamine electrodes cited above suggest the technology has progressed and that a stable histamine electrode similar to the current glucose electrodes may be in sight.

Robert Bateman
Principal Investigator